Remarks

Applicants request entry of the amendments and reexamination of the application. Applicants also enclose a Request for Extension of Time in order to enter this paper.

Claim 65 has been amended to clarify the "expression cassette" recitation, as requested by the Examiner (*see* page 3 of Paper No. 20). Amended claim 65 now recites an expression cassette having the coding sequences of two different neurotrophic factors. Claims 67-69 have been amended to clarify the recitation of adenovirus vector, as requested by the Examiner (*see* page 3 of Paper No. 20). The phrase "recombinant adenoviruses" has been replaced with "recombinant adenovirus vectors," for example. Claims 92 and 113 have been amended to recite "a bicistronic expression cassette." This amendment clarifies the language the Examiner referred to in claims 92-96 and 113 (*see* page 3 of Paper No. 20). Furthermore, the specification refers to a bicistronic unit at page 12, lines 9-17, thus clearly and unequivocally describing a bicistronic approach to expressing two or more sequences now recited in the claims.

Claim 95 has been amended to refer to the bicistronic expression cassette of claim 92.

No new matter enters by these amendments.

Objection to the Drawings

The Advisory Action (Paper No. 25) indicated that the drawing corrections submitted on July 28, 2003, are approved.

Rejection under 35 U.S.C. § 112, Second Paragraph

The sole remaining rejection to the application is a rejection of claims 65, 67-69, 92-96, and 113, under 35 U.S.C. § 112, second paragraph. The PTO asserts that these

claims are indefinite for failing to particularly point out and distinctly claim the subject matter claimed. Applicants respectfully disagree.

As explained in the response filed November 12, 2002, the claims that include references to adenoviruses and expression cassettes are clearly understood by one of ordinary skill in the art. However, solely to expedite the prosecution of this case and because none of the amendments restrict or limit the scope of the claims, applicants have proposed several changes to the language. These changes simply change the reference to the vectors from "recombinant adenoviruses" to "recombinant adenoviral vectors" in claims 67-69. Since the prior use of the term "adenoviruses" included the understanding that the viruses were being used as vectors for the encoded neurotrophic factors, this amendment merely uses alternative language.

Also, claim 65 has been amended to clarify that the vector encodes the neurotrophic factors, as one of skill in the art would have understood from the relationship to claim 64, which recites that the two vectors encode neurotrophic factors. Applicants have also changed the language relating to the simultaneous expression of the neurotrophic factors to make it consistent with the rest of the amended claim. Pages 7-14 of the specification, for example, describe the many options one of ordinary skill in the art may select from and use to prepare the adenovirus vectors recited in the claims, and page 12 specifically refers to options in preparing vectors for simultaneous expression.

Finally, claims 92 and 113 have been amended to add the term "bicistronic." As explained above, the specification specifically refers to this option at page 12, lines 9-17. Applicants submit that one of ordinary skill in the art understands the reference to bicistronic expression cassettes and would have had the same understanding at the time the application was filed. In support of this statement, applicants submit several journal articles and abstracts that specifically refer to bicistronic expression and the use of vectors with bicistronic constructs at the relevant time period. For example, Castleden *et al.* (abstract only) refer to bicistronic constructs in retroviral and adenoviral vectors, Chen *et al.* (abstract only) refer to a bicistronic adenoviral vector, Kulmburg *et al.* (J. Mol. Med. 75:223-228) refer to bicistronic plasmid vectors, Havenga *et al.* (abstract only) refers to the general

concept of bicistronic constructs, and Lee *et al.* (J. Virol. 72: 8430-8436) refers to bicistronic plasmid vectors. Applicants have enclosed copies of these documents for the Examiner's convenience. Clearly, one of ordinary skill in the art was familiar with the general concept of bicistronic cassettes or constructs to express sequences and was familiar with how to make, use, and prepare these bicistronic cassettes. Therefore, taking into account the knowledge, skill, and experience of one of ordinary skill at the time of the invention, applicants' statements in the specification referring specifically to bicistronic expression adequately define and describe the vectors recited in the claims.

Applicants respectfully request withdrawal of this rejection.

Conclusion

Applicants respectfully submit that this application is in condition for allowance. If the Examiner believes that an interview with Applicant's representative, either by telephone or in person, would further prosecution of this application, we would welcome the opportunity for such an interview.

Applicants have included a Request for Extension of Time necessary to enter this amendment. No additional extension of time fees, requests for extension of time, other petitions, additional claim fees, or other fees are believed to be necessary to enter and consider this paper. If, however, any extensions of time are required or any fees are due in order to enter or consider this paper or enter or consider any paper accompanying this paper, including fees for net addition of claims, applicants hereby request any extensions or petitions necessary and the Commissioner is hereby authorized to charge our Deposit Account No. 50-1129 for any fees. If there is any variance between the fee submitted and any fee required, or if the payment or fee payment information has been misplaced or is somehow insufficient to provide payment, the Commissioner is hereby authorized to charge or credit Deposit Account No. 50-1129.

Respectfully submitted,
WILEY REIN & FIELDING LLP

Date: Oct. 2, 2003

By: David J. Kulik Reg. No. 36,576

Enclosure

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1: Hum Gene Ther. 1997 Nov 20;8(17):2087-102. Related Articles, Links

A family of bicistronic vectors to enhance both local and systemic antitumor effects of HSVtk or cytokine expression in a murine melanoma model.

Castleden SA, Chong H, Garcia-Ribas I, Melcher AA, Hutchinson G, Roberts B, Hart IR, Vile RG.

Imperial Cancer Research Fund Laboratory of Molecular Therapy, Royal Postgraduate Medical School, Hammersmith Hospital, London, UK.

The herpes simplex virus-thymidine kinase/ganciclovir (HSVtk/GCV) system produces both direct and immune-mediated tumor cell killing. Here, we compare the efficacy of HSVtk/GCV with cytokines, alone and in combination, on the tumorigenicity and immunogenicity of B16 cells. With respect to single gene modifications, only HSVtk/GCV, or high-level interleukin-2 (IL-2) secretion, completely prevented tumor growth, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) generated the best levels of long-term systemic protection. To augment both local killing and immune activation, we constructed bicistronic constructs that express HSVtk and a cytokine within the same cell. Co-expression of HSVtk with IL-2 or GM-CSF enhanced the local antitumor activity of any gene alone. In a tumor-prevention model, HSVtk killing, in an environment preprimed with GM-CSF, generated the best long-term immune protection. However, in a short-term therapy model, continued IL-2 expression was most effective against 3-day established tumors. This probably reflects differences in the activities of IL-2 and GM-CSF in generating short-term, nonspecific immune stimulation compared to long-term immunological memory, respectively. As a prelude to in vivo delivery experiments, we also demonstrated that these bicistronic cassettes can be packaged normally into retroviral (5 x 10(5) virus/ml from pooled populations) and adenoviral vectors (5 x 10(9) virus/ml) and function as predicted within virally infected cells. This family of bicistronic vectors can be used to stimulate synergy between suicide and cytokine genes, overcomes the problems of delivering two genes on separate vectors, and should allow easier preparation of vectors for the delivery of multiple genes to patients' tumor cells.

PMID: 9414257 [PubMed - indexed for MEDLINE]

1: J Immunol. 1997 Jul 1;159(1):351-9.

Related Articles, Links

Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cDNAs for the IL-12 heterodimer and its inhibition by the IL-12 p40 subunit homodimer.

Chen L, Chen D, Block E, O'Donnell M, Kufe DW, Clinton SK.

Dana-Farber Cancer Institute, Department of Urology, Harvard Medical School, Boston, MA 02115, USA.

IL-12 is a heterodimeric immunoregulatory cytokine composed of covalently linked p40 and p35 subunits and exhibits antitumor activity in a variety of laboratory models. The efficacy of systemically administered cytokines for cancer therapy is often limited by toxicity. The gene therapy approach provides a mechanism to achieve temporary and high local concentrations of cytokines within a tumor with less risk of systemic toxicity. We constructed replication-defective adenoviruses containing the murine IL-12 p40 subunit (Ad.mp40) or a bicistronic vector containing cDNAs for the p40 and p35 subunits (Ad.mIL-12). Murine MB49 bladder cancer cells infected with Ad.mIL-12 secrete high concentrations of biologically active IL-12, while those infected with Ad.mp40 produce the p40 homodimer. Tumors injected with Ad.mIL-12 show rapid increases in IL-12 and IFN-gamma expression over 2 to 5 days and a return to baseline by 7 to 14 days. Injection of tumors with Ad.mIL-12 (1 x 10(9) plaque-forming units) results in a complete tumor regression in all mice, while those treated with control adenovirus succumb to their tumor. Efficacy is reduced when studies are performed in mice depleted of CD4+ and CD8+ cells or in nude mice. Mice cured of their tumor by Ad.mIL-12 demonstrate specific protective immunity upon rechallenge. Ad.mp40 does not exhibit antitumor activity and may antagonize the activity of rIL-12 or Ad.mIL-12. In summary, gene therapy strategies for cancer using adenoviral vectors containing IL-12 are highly effective with no significant toxicity in mice.

PMID: 9200473 [PubMed - indexed for MEDLINE]

J Mol Med (1997) 75:223–228

ORIGINAL ARTICLE

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NTS influence on transgene expression from mono- and bicistronic plasmid vectors after lipofection in a human fibroblast cell line

Received: 1 October 1996 / Accepted: 27 November 1996

Abstract We evaluated the survival, transgene production, and copy numbers of integrated plasmid units per host genome after lipofection with mono- and bicistronic plasmid vectors in different cell lines and under various conditions. The addition of an integration enhancing murine sequence nontranscribed spacer (NTS) to the plasmids increased transfection efficiency, survival, and transgene expression. However, in human fibroblast cells this sequence had only marginal effects on overall plasmid copy number in bulk cultures. Clones producing the highest amounts of the transgene contained only one or two copies of plasmid per genome, independent of cell type and plasmid design.

Key words Plasmid vector · Lipofection · Transgene expression · Gene therapy · Nontranscribed Spacer

Abbreviations *CMV* Cytomegalovirus · *G-CSF* Granulocyte-colony stimulating factor · *IRES* Internal ribosomal entry sequence · *NTS* Nontranscribed spacer

Introduction

To improve gene therapy it is important to enhance the integration of foreign DNA into suitable cells and to ensure a high level of transcription of the transfected transgene. While retroviral gene transfer with its comparatively high transduction efficiency offers many advantages, nonviral plasmid vector systems can be used effectively ex vivo when cultured cells are used that can be selected and cloned according to criteria considered optimal for in vivo therapy upon administration of transfected cells [1–6]. To optimize gene transfer and transcription of recombinant genes in a plasmid-based gene transfer model we investigated the effects of selection pressure, copy number, and various vector backbones on transfection and transcription.

Several events can result in high expression of the plasmid's transcriptional units. First, more than one copy of plasmid DNA per cell might in some systems assure more transcriptional units leading to higher protein production per cell. Second, integration can occur at a very active region of the chromosome and a plasmid-borne promoter acitvity can be multiplied by cellular enhancers. Third, the plasmid itself activates via plasmid-encoded sequences the chromosome at the locus of integration and thus activates transcription per se.

We designed a variety of plasmids addressing these questions. The plasmids contained a selectable neomycin resistance gene marker [7], a granulocyte colony stimulating factor (G-CSF) expression cassette [8], and a nontranscribed spacer (NTS) sequence. This NTS sequence derived from murine rDNA is apparently involved in nucleosome assembly and plasmid amplification. Plasmids containing this sequence have been shown both in murine and in some human cells to raise integration at high copy numbers [9, 10].

We investigated the effects of the NTS sequence on transgene expression, studying both the quantity of aminoglycoside phosphotransferase gene (neomycin resistance) expression at increasing neomycin concentrations and the expression of the gene of interest by quantitating the gene product by enzyme-linked immunosorbent assay. G-CSF was chosen as the gene of interest in view of its ability to ameliorate chemotherapy-induced cytopenia in an in vivo model system [11]. Since we have previously shown that an internal ribosomal entry sequence (IRES) [12, 13] allowing the translation of the G-CSF cDNA and the neomycin resistance gene from the same RNA, tightly linking the two genes, leads to enhanced transgene expression [14], we investigated the effect of

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the addition of the NTS sequence on the first generation vectors (two cassettes) and on the second generation vectors containing the IRES sequence.

The rationale was to combine three different approaches to obtain high producer clones from human fibroblasts: (a) the NTS sequence in our vectors should allow plasmid amplification or local chromosomal structure change, (b) selection pressure was employed by increasing neomycin concentrations in the culture media to select highly active neomycin transcription units, (c) we coupled the expression of neomycin resistance directly to the expression of the transgene by using an IRES vector.

Our results indicate that the NTS sequence in combination with high neomycin doses does increase the probability of obtaining high producer clones. The direct linkage of transgene expression to the expression of the selection marker via the IRES sequence additionally improves transgene expression. We describe here an easy-to-clone plasmid-based vector system with improved transfection efficiency and high expression rate of the transgene.

Material and methods

Murine CMS-5 fibrosarcoma cells, Balb3T3 fibroblasts, and human immortalized KMST-6 fibroblasts [15] were grown in high-glucose Dulbecco's modified Eagle's medium (Gibco, Scotland) supplemented with 10% fetal calf serum (PanSystems, Germany), 2 mM glutamine, 2 mM sodium pyruvate, and 50 µg/ml gentamycin.

pCMV-GCSF#Neo contains a human G-CSF cDNA under control of the human cytomegalovirus (CMV) immediate/early promoter and a polyadenylation signal derived from SV40. It carries in addition a second expression cassette for aminoglycoside phosphotransferase under the control of the TK promoter and polyoma enhancer (Gift from T. Boehm). pCMV-GCSF-IRES-TkNeo and pActin-GCSF-IRES-TkNeo express the human G-CSF cDNA linked to the IRES-TKNeo cassette (Gift from H. Veelken) under the human CMV or human β-actin promoter (Gift from P. Fisch), respectively. The NTS fragment was a gift from F. Grummt. Plasmids are shown in Fig. 1. All plasmids used for transfections of eukaryotic cells were purified by anion exchange chromatography (Macherey & Nagel, Düren, Germany).

The quantity of 4 µg plasmid DNA in 50 µl 0.9% NaCl and 12 µl Transfectam (Promega, Germany) in 50 µl 0.9% NaCl were vortexed, left for 10 min at 25°C, then mixed and incubated again for 10 min at 25°C to

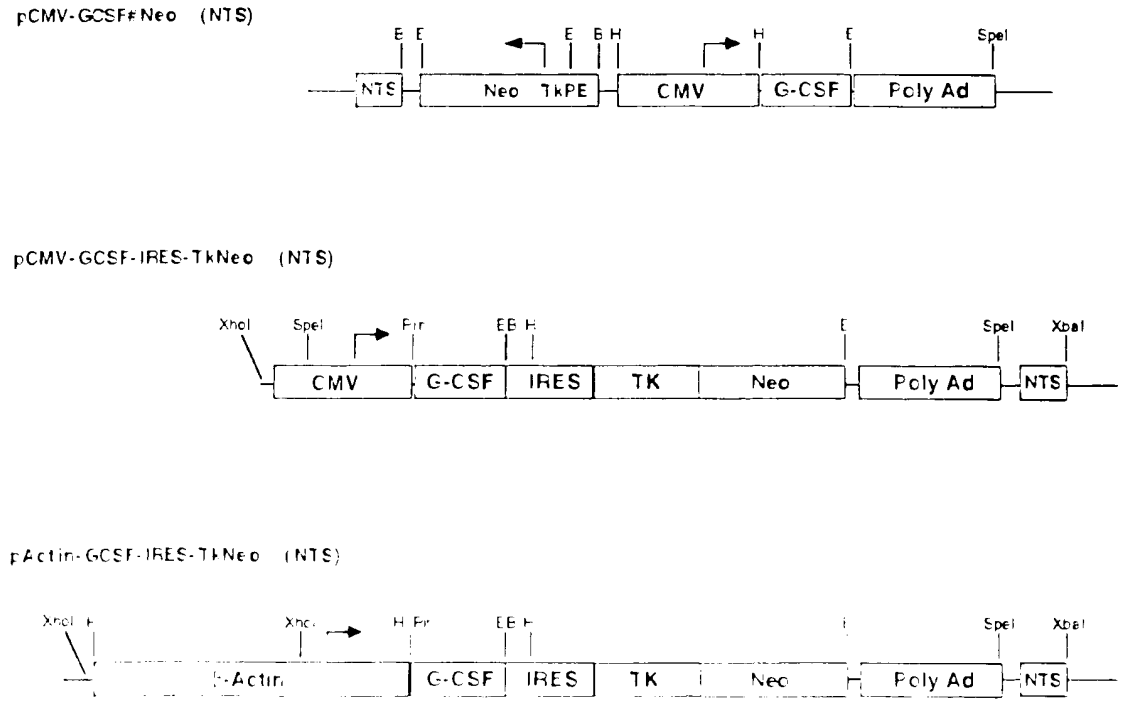


Fig. 1 Plasmid expression vectors presented in this paper. pCMV-G-CSF#Neo contains two independent expression cassettes for the aminoglycoside phosphotransferase (*Neo*) under control of the thymindin kinase promoter + polyoma enhancer (*TkPE*) and for G-CSF under control of the CMV promoter. pCMV-GCSF-IRES-TkNeo and pActin-GCSF-IRES-TkNeo contain one single expression cassette linking the thymidine kinase (*Tk*)–aminoglycoside phosphotransferase fusion protein via an internal ribosomal entry sequence (*IRES*) directly to the G-CSF cDNA. This cassette is under the control of the CMV or human β-actin promoter, respectively. All vectors harbor the SV40 polyadenylation signal (*Poly Ad*). *Arrows*, the sens of transcription. NTS-plasmids contain the murine NTS sequence at the indicated positions. Abbreviations for restriction enzyme recognition sequences: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *Pin*, *Pin*AI

form the transfection complex. Cells (10^5) that were plated in 35-mm tissue culture dishes 1 day prior to transfection, washed twice with serum-free medium before transfection, and overlayed with 1 ml of serum-free medium. The transfection complex was added to the cells and the mixture incubated at 37°C at 5% CO_2. After 2 h 1 ml of regular growth medium containing 20% fetal calf serum was added, and after 24 h, the entire culture supernatant was replaced with fresh medium. Selection with G418 (Gibco, Germany) was started the next day.

Defined supernatants were generated by plating 10^4 cells/cm^2 into tissue culture flasks. On the following day the growth medium was replaced completely. After 24 h supernatants were collected, and cells were counted. Concentrations of human G-CSF were determined using commercially available enzyme-linked immunosorbent assays (R&D Systems, Germany).

Chromosomal DNA from cells was obtained using the QIAamp Tissue Kit (Qiagen, Germany) according to the standard protocol given in the kit. DNAs were slot blotted on a nylon membrane (MilliBlot-S, MilliPore, Germany) and hybridized with a ^{32}P-labeled probe corresponding to the coding region of the aminoglycoside phosphotransferase gene under stringent conditions [16]. After exposure on a Phospholmager FUJIX BAS 1000 (Fuji, Germany), the membranes were rehybridized with probes corresponding to the promoter regions of human β-actin or human collagen I α_1 to verify equal distribution of the DNA transferred to the membrane. The KMST-6/14 clone was used as a single copy control (H. Veelken, unpublished data).

Transfection efficiencies were analyzed with a Poisson regression model with a log LINK function [17] using the program Genmod in SAS for the number of colonies. G-CSF production differences in murine cells were evaluated by a two-tailed Wilcoxon test [18] using the program StatXact. The effect of the NTS sequence on G-CSF production in KMST-6 cells at various neomycin concentrations was analyzed by a logistic regression model [19] using the LOGISTIC procedure in SAS. The success criterion was production of a considerable amount of G-CSF (>0 for pCMV-GCSF#Neo transfected cells and > 100 ng for pActin-GCSF-IRES-TkNeo transfected cells, respectively). Copy number differences were compared by a two-tailed Wilcoxon test.

Results

Transfection efficiency and expression of the neomycin resistance gene (aminoglycoside phosphotransferase)

Balb3T3, CMS-5, and KMST-6 were lipofected with 4 µg plasmid vector in an uncut CCC form. Primary results had shown no effect on transfection efficiency or transgene expression regardless of whether the plasmid was linearized prior to transfection (data not shown). After transfection the cells were selected at 500 µg/ml neomycin for 14 days, then colonies were counted under the microscope. Only colonies larger than 20 cells were counted (Table 1).

Primary selection is for the expression of the selection marker and not against the transgene. The number of stably transfected colonies obtained is a good means to measure the quantity of expression of the selection marker at increasing concentrations of neomycin. In the next experiment we transfected 10^5 KMST-6 cells with 4 µg plasmid and selected clones at various neomycin concen-

Table 1 Colony numbers obtained in different cell lines with various plasmids with and without NTS at increasing neomycin concentrations

Plasmid	NTS	Cell line							
		Balb3T3		CMS-5		KMST-6			
		Colonies	Neomycin (µg/ml)	Colonies	Neomycin (µg/ml)	Colonies	Neomycin (µg/ml)	+NTS/−NTS ratio	*P* value (per group)
pCMV-GCSF#Neo	−	500–800	500	350–500	500	90–110	500		
pCMV-GCSF#Neo	−	500–800	500	350–500	500	150–160	500	1.5	
pCMV-GCSF#Neo	−					36	1000		
pCMV-GCSF#Neo	−					124	1000	3.4	
pCMV-GCSF#Neo	−					12	2000		
pCMV-GCSF#Neo	+					52	2000	4.3	
pCMV-GCSF#Neo	−					4	3000		
pCMV-GCSF#Neo	−					28	3000	7	<0.0001
pCMV-GCSF-IRES-TkNeo	−					100–125	500		
pCMV-GCSF-IRES-TkNeo	+					150–175	500	1.5	<0.0001
pActin-GCSF-IRES-TkNeo						90–105	500		
pActin-GCSF-IRES-TkNeo	−					240–265	500	2.5	
pActin-GCSF-IRES-TkNeo						58	1000		
pActin-GCSF-IRES-TkNeo	−					137	1000	2.4	
pActin-GCSF-IRES-TkNeo						21	2000		
pActin-GCSF-IRES-TkNeo						85	2000	4	<0.0001

Table 2 G-CSF production by selected clones in various cell lines transfected with plasmids with and without NTS

Plasmid	NTS	Cell line	Neomycin (μg ml)	G-CSF (μg 24 h)			*P* value (±NTS)
				Median	Range	Mean±SD	
pCMV-GCSF±Neo	-	Balb3T3	500	0	0–10	2.9±4.5	<0.028
pCMV-GCSF±Neo	-	Balb3T3	500	25	0–85	30.0±32.8	
pCMV-GCSF±Neo	-	CMS-5	500	0	0–35	3.8±9.8	<0.0006
pCMV-GCSF±Neo	-	CMS-5	500	10	0–110	18.6±27.4	
pCMV-GCSF±Neo	-	KMST-6	500	0	0–22.6	5.6±9.0	
pCMV-GCSF±Neo	-	KMST-6	1000	0	0–5.3	1.4±2.0	
pCMV-GCSF±Neo	-	KMST-6	2000	0	0	0.0±0.0	
pCMV-GCSF±Neo	-	KMST-6	3000	0	0–0.5	0.2±0.2	<0.013
pCMV-GCSF±Neo	-	KMST-6	500	0	0–21.2	3.7±6.8	
pCMV-GCSF±Neo	-	KMST-6	1000	5.45	0–56.5	13.5±19.7	
pCMV-GCSF±Neo	-	KMST-6	2000	10.7	0–68.8	14.8±21.3	
pCMV-GCSF±Neo	-	KMST-6	3000	19.9	0–100	27.5±32.2	
pActin-GCSF-IRES-TkNeo	-	KMST-6	500	45	7–215	58.8±55.9	
pActin-GCSF-IRES-TkNeo	-	KMST-6	1000	26	0–171	43.5±48.7	
pActin-GCSF-IRES-TkNeo	-	KMST-6	2000	35	0–196	47.9±49.1	<0.047
pActin-GCSF-IRES-TkNeo	-	KMST-6	500	41.5	0–161	55.7±51.7	
pActin-GCSF-IRES-TkNeo	-	KMST-6	1000	27	1–415	77.0±111.5	
pActin-GCSF-IRES-TkNeo	-	KMST-6	2000	73	15–304	107.6±94.1	

trations for 14 days. Colonies were counted again as above (Table 1). Selection was performed using increasing concentrations of neomycin in the media during growth of transfected cells. Preliminary tests showed the effectiveness of this approach in murine bulk cultures since a good correlation between increasing neomycin concentration and G-CSF production could be observed (data not shown).

The addition of the NTS sequence increased the transfection efficieny in the human system (KMST-6) by 50%–700%. Since the transfection efficiency in the murine system was already very high (up to 8% stably transfected clones), no further statistically significant increase was observed. As shown in Table 1, colony numbers found with KMST-6 cells drop when higher selection pressure is administered. This indicates that some of the transfectants produce insufficient aminoglycoside phosphotransferase to survive under high selection pressure. These experiments showed that cells transfected with the plasmids containing the NTS sequence have higher resistance gene expression as shown by significantly better survival ($P<0.0001$) than the transfectants without the NTS sequence. On the other hand, the vector containing in addition the IRES sequence gave the highest number of neomycin-resistant clones. Furthermore, NTS-containing colonies do grow better and more quickly than their non-NTS counterparts (data not shown).

Expression of the transgene G-CSF: effect of NTS

We next tested expression of the transgene of interest. Balb3T3 and CMS-5 cells were transfected with 4 μg plasmic DNA. Stably transfected colonies were selected at 500 μg ml neomycin. KMST-6 cells were transfected with the first-generation vector and with the IRES construct and selected at various neomycin concentrations. Colonies were ring-cloned after two weeks and further expanded in media containing 500 μg/ml neomycin. The G-CSF content of the media was then measured by enzyme-linked immunosorbent assay (Table 2).

As demonstrated before (Table 1), the strongest impact of the NTS sequence was on the level of aminoglycoside phosphotransferase expression, seen at high neomycin concentrations. We thus investigated the role of the NTS sequence in transfection experiments in human fibroblasts, with selection at increasing neomycin concentrations. We wanted to determine whether increased expression of the selection marker is associated with high expression of the transgene as well. As shown in Table 2, the NTS sequence generates clones at high neomycin concentrations with the highest levels of G-CSF expression. The effect of NTS was statistically significant with both types of plasmids, although the effect was less pronounced in cells harboring the IRES construct. This may be due to the fact that the genes behind an IRES are expressed less than the genes upstream of the IRES [20]. This means that in the IRES construct neomycin resistance may already be a limiting factor, imitating a high selection pressure. This hypothesis is supposed by the observation that no stable clones can be obtained at 3000 mg ml neomycin using the IRES construct.

Comparing the first-generation vector and the IRES construct, it is clear that the IRES construct yields the best results. Since expression of the transgene is linked to expression of the selection marker gene, none of the tested clones lacks expression of the transgene. As the selection marker is probably expressed only at lower levels than the independent cassette due to its position behind the IRES, the effect of rising transgene expression with increasing neomycin concentration is less pronounced.

Copy number

The NTS sequence was originally described in the murine system to increase the copy number of integrated plasmids in the genome. No correlation, however, was demonstrated between actual copy number and level of transgene expression. Other mechanisms, such as local chromosome conformation changes, have been proposed as well.

In a final set of experiments, we sought to clarify which of the proposed mechanisms accounts for improvement in aminoglycoside phosphotransferase and G-CSF production in clones transfected with the NTS-plasmids and selected at high neomycin concentration: high copy number or a local activation of the chromosome by the NTS sequence? To distinguish between these two mechanisms, we estimated copy numbers of the integrated plasmid in bulk cultures and selected clones. Chromosomal DNA was isolated from expanded cells and slot blotted at equal amounts onto a nylon membrane. We estimated effective copy numbers by comparison of radioactivity hybridized to the slot-blotted DNAs and scanned with a PhosphoImager.

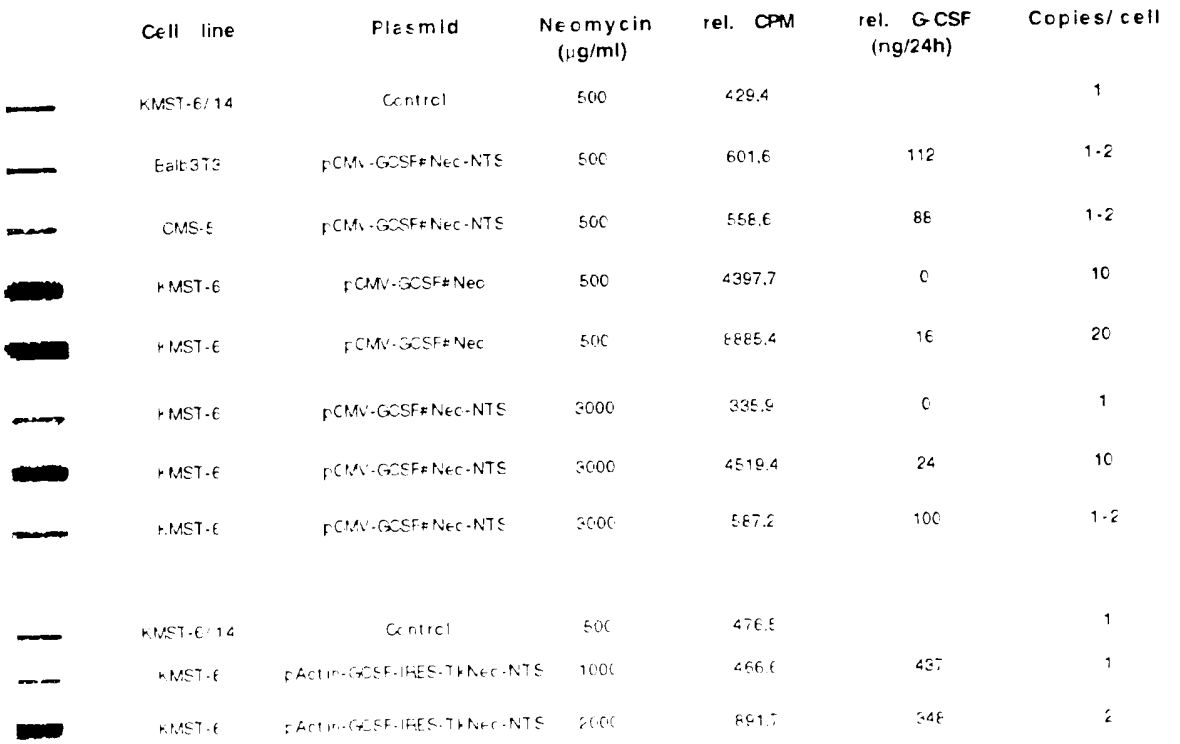
As a probe we chose the coding region of the aminoglycoside phosphotransferase gene (neomycin resistance). For a positive control for single copy integration into the KMST-6 genome, we used DNA from a reference clone (KMST-6/14). This clone has been shown by Southern blot analysis to contain only a single copy of the aminoglycoside phosphotransferase gene integrated (H. Veelken, unpublished results). To exclude variations in DNA load, the blots were rehybridized with a fragment from the human collagen I α_1 promoter (data not shown), and radioactivity counts from the neomycin hybridization were standardized against these values.

Table 3 Copy numbers found in KMST-6 bulk cultures

Neomycin (μg ml)	Copy number obtained with plasmid			
	pCMV-GCSF#Neo	pCMV-GCSF#Neo-NTS	pActin-GCSF-IRES-TkNeo	pActin-GCSF-IRES-TkNeo-NTS
500	8.1	9.1	1.5	2.3
1000	7.5	11.5	1.4	2.1
2000	7.3	10.7	1.6	2.3
3000	7.2	9.4	nd	nd
	P=0.029 (±NTS)		P=0.1[a] (±NTS)	

[a] Few data points

Fig. 2 Copy number detection of plasmid DNA in selected clones. Chromosomal DNA from cells was slot blotted on a nylon membrane and hybridized with a ^{32}P-labeled probe corresponding to the coding region of the aminoglycoside phosphotransferase gene (neomycin resistance). Filters were exposed on a PhosphoImager to count radioactivity in the hybridization areas. The membranes were then re-hybridized with probes corresponding to the promoter regions of human β-actin or human collagen I α_1 and recounted on the PhosphoImager to verify equal distribution of the DNA transferred to the membrane. Corrected radioactivity counts are given as relative counts per minute (*rel. CPM*). The clone KMST-6/14 was shown previously by Southern blotting to contain a single plasmid copy harboring one unit of the aminoglycoside phosphotransferase gene



Copy numbers in KMST-6 bulk cultures were determined and found to be elevated in cells transfected with the NTS-plasmids about 1.5-fold, as described in Table 3. Copy numbers were higher in bulk cultures obtained from transfections with NTS-containing plasmids; nonetheless, the increase was only marginal (1.5-fold). Interestingly, the IRES plasmids give rise to only very low copy numbers, suggesting a selection of integration sites allowing high transgene expression.

We then concentrated on selected clones from the above experiments. We analyzed the best G-CSF producing clones obtained from Balb3T3, CMS-5, and KMST-6 cells. We added zero and intermediate producers from KMST-6 cell transfections, obtained at 500 and 3000 μg/ml of neomycin, respectively. As with the bulk cultures, chromosomal DNA was isolated from the clones, blotted to nylon membranes and hybridized with the aminoglycoside phosphotransferase-specific probe. Equal DNA load was checked by rehybridization with a probe corresponding to the human β-actin or human collagen I α_1 promoter, respectively (data not shown).

Figure 2 shows the scanned autoradiogram of DNAs obtained from selected clones and hybridized with the neomycin probe. Relative counts are corrected to DNA content. KMST-6/14 is used as a single-copy control. All high producer clones from any cell line contained only very low (1 or 2) copies of plasmid integrated. Nevertheless, higher copy numbers (10–20) were seen in other clones producing intermediate amounts of G-CSF or no G-CSF. Taken together, bicistronic IRES constructs containing the NTS sequence are very potent transfection vectors, combining the advantage of high transfection efficiency with high expression of the transgene. The vectors are cloned in a way that new transgene cDNAs can be inserted easily using the unique restriction sites *Pin*AI and *Bam*HI; new promoters can be inserted after restriction of the vectors with the single cutters *Xho*I and *Pin*AI (Fig. 1).

Discussion

Gene therapy using transfection systems involving plasmid vectors is hampered by the problem that individual clones vary broadly in their production of the desired transgene product. High numbers of clones must therefore be screened to find a suitable producer clone. Our goals were to establish a method that would allow a more rapid screening for clones and to enhance production of the transgene.

We attempted to improve expression of G-CSF from murine and human fibroblast cell lines by an optimized selection procedure. We selected clones that expressed high neomycin resistance and thus could survive under high selection pressure. Three events may influence this result: First, the plasmid may integrate at high copy number. As described for gene amplification in the case of the dehydrofolate reductase gene under methotrexate selection, a multitude of transcriptional units would provide a high resistance level. Second, the plasmid may integrate in the chromosome into a transcriptionally active locus and profit from adjacent cellular enhancers. Third, the plasmid may integrate at random but induce by sequences provided by itself, for example, the NTS sequence, a conformational change in the chromosome's structure leading to high transcriptional activity. At the level of vector design we tested monocistronic and bicistronic plasmids. The latter directly link expression of the transgene to the expression of the selection marker gene.

Introduction of the NTS sequence into the vector leads to an improvement in transfection efficiency and significantly increases G-CSF production with both first- and second-generation vectors. These results are in concordance with the NTS systems tested and described in the literature [10]. Best results were obtained in human cells when combining the two approaches, NTS-containing vectors and selection at high neomycin concentrations.

In contrast, we observed differences in the way in which the NTS sequence affects gene expression. Although data from the literature [10] suggest that improvement is primarily based on the increase in active vector copies, we found no evidence for this principle in our human fibroblast system. Copy numbers were higher in bulk cultures transfected with plasmids containing the NTS sequence but the effect on transgene expression was only marginal (less than twofold increase).

Clones selected for producing high amounts of the transgene G-CSF had only low copy numbers (one or two) of plasmid integrated into the genome. Since this was reproducible in various cell lines transfected with different plasmids, we suggest that high copy numbers do not provide an advantage in our system. A single well-placed copy of the plasmid may act more efficiently than several hundred copies arranged in a head-to-tail array [21] but inserted at the wrong place. The NTS sequence in our system seems to enhance integration at an active chromosomal site or activates the integration site by its ability to bind high mobility group proteins known to influence chromsomal structure [9].

This is important with regard to insertional mutagenesis. If the plasmids were forced to integrate into already active parts of the chromosome, they could interfere with transcription of important cellular genes. We would then observe changes in growth characteristics. The fact that most NTS-less pCMV-GSCF#Neo transfected cells selected at high neomycin concentrations die could be an indicator for such events. In contrast, no change in growth characteristics or morphology has been seen in clones transfected with pCMV-GSCF#Neo containing the NTS sequence. These data suggest that no important genes have been injured during the transfection event, and that the NTS sequence improves transcription and production rather by activating the integration site than by interference with already active loci on the chromosome.

We have shown that increasing concentrations of the selection agent neomycin in growth media is associated

with improved transgene expression. This effect was more pronounced in cells transfected with the first-generation vector than in cells transfected with the second-generation IRES vectors. It has been shown [20] that genes translated behind an IRES sequence are three to ten times less efficiently translated than those translated directly by the normal CAP-dependent mechanism used by eukaryotic cells in general. Very probably our IRES-containing vectors therefore show self-restriction in the expression of the aminoglycoside resistance gene and internally increase the production of the transgene.

Using high amounts of neomycin in the medium is not only costly, but high expression of the aminoglycoside resistance gene may have negative effects on the cell itself [22]. Using the second-generation IRES plasmids offers the advantage of internal limitation of the aminoglycoside resistance gene overexpression combined with an enhanced transgene expression. The addition of the NTS sequence increases the probability of obtaining a highly active plasmid copy in the genome. Since the internal linkage of the transgene to the selection marker eliminates virtually all nul-producer clones, the combination of NTS and IRES background seems to be the vector of choice for the expression system described achieving high transfection efficiency and increased transgene expression at low selection toxicity in human fibroblasts.

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Related Articles, Links

Second gene expression in bicistronic constructs using short synthetic intercistrons and viral IRES sequences.

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In this study, we describe the efficiency of second gene translation in bicistronic constructs containing either a short (36bp) synthetic intercistron or known internal ribosomal entry sites (IRES). Experiments were performed using two different gene combinations: Herpes simplex virus-thymidine kinase (HSV-TK) and neomycine (NEO) or human glucocerebrosidase (hGC) and a methotrexate (MTX) resistant mutant dihydrofolate reductase (DHFR). We demonstrate that upon transfection, second gene translation is efficient using either an IRES or a 36-bp intercistron. Infection with retrovirus carrying the TK and NEO genes linked via a 36-bp intercistron resulted in both G418R (NEO expression) and gancyclovir (GCV) sensitivity (TK expression), indicating that both genes were expressed and thus that the genomic DNA and RNA of this bicistronic construct were intact. Likewise, retrovirus carrying the hGC and mutant DHFR gene separated by a short intercistron was harvested from MTXR murine PsiCRE cells. However, infection of PA317 cells with this virus supernatant did not result in the presence of hGC enzyme activity in these murine cells. Proviral DNA and RNA analyses indicated that the hGC coding region was lost from the original construct in the infected PA317 cells. In contrast, retrovirus carrying the hGC and DHFR cDNAs was linked via an IRES functioned as expected. Based on these results, we conclude that the efficiency of second gene translation using short synthetic intercistrons might prove useful in bicistronic constructs, depending on the gene combination used.

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Optimal Induction of Hepatitis C Virus Envelope-Specific Immunity by Bicistronic Plasmid DNA Inoculation with the Granulocyte-Macrophage Colony-Stimulating Factor Gene

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In this study, we have constructed various DNA vaccine vectors that carried hepatitis C virus (HCV) envelope genes without and with the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene in several different ways. In Buffalo rats that received plasmids carrying the HCV envelope genes, which encode envelope proteins E1 and E2, both antibody and lymphoproliferative responses against these proteins were induced. These responses were greatly enhanced by the codelivery of the GM-CSF gene. In particular, inoculation with a bicistronic plasmid that independently expressed the GM-CSF gene and the envelope genes in the same construct generated the highest antibody titers and significantly increased lymphoproliferative responses against these proteins. Moreover, strong antibody responses to homologous and heterologous hypervariable region 1 peptides were elicited in the immunized rats.

Hepatitis C virus (HCV) has been identified as a major causative agent of posttransfusion and sporadic non-A, non-B hepatitis (2, 13). More than 70% of HCV infections are persistent and eventually lead to liver cirrhosis and hepatocellular carcinoma (28). To date, the only treatment for chronic HCV infection is alpha interferon therapy. However, long-term responses to this therapy occur in only 10 to 30% of patients (20, 25). Therefore, the development of a vaccine to prevent HCV infection is of the greatest urgency. HCV has a 9.5-kb positive-strand RNA genome that encodes a single polypeptide. The polypeptide is processed by cellular and viral proteinases to produce both the structural and the nonstructural HCV proteins (4, 10, 30). Based on data that was derived from clinical and experimental studies of humans and chimpanzees, it has been suggested that both humoral and cellular immune responses to HCV proteins can be generated (8, 11, 24, 26). It has been shown that HCV envelope proteins 1 and 2 appear to be key viral antigens for the induction of protective immunity in experimental chimpanzees (3). Recently, DNA vaccine approaches have been applied to generate immunity to HCV proteins. The expression of the HCV core and E2 proteins resulted in the generation of HCV antigen-specific immune responses (14, 19, 21, 33).

The use of cytokines to modulate immune responses in DNA immunization is being actively investigated. Granulocyte-macrophage colony-stimulating factor (GM-CSF), a hematopoietic growth factor, has been widely used as a molecular adjuvant to induce immunity. It has been shown that idiotype-GM-CSF fusion proteins are effective vaccines for lymphoma, without the need for another adjuvant (32). In addition, the intramuscular inoculation of the GM-CSF gene together with plasmids carrying viral genes, such as those encoding the glycoprotein of rabies virus and VP1 of encephalomyocarditis virus, increased antigen-specific immune responses and protective immunity (31, 36). Other cytokines such as interleukin-2, interleukin-12, and gamma interferon have also been shown to enhance the immune responses to coadministered antigens (5, 12, 37). These reports suggest that the local expression of relevant cytokine genes can affect the microenvironment, which allows for immune responses to be elicited by the coadministered antigens.

In this study, we compared the levels of immune responses induced by HCV E1 and E2 DNA-based immunization without and with various forms of the GM-CSF gene in Buffalo rats. Our result demonstrated that HCV envelope-specific immune responses were significantly enhanced by the codelivery of the GM-CSF gene. The coexpression of the GM-CSF and HCV envelope proteins from a bicistronic vector most effectively generated envelope-specific antibodies and lymphoproliferative responses. Furthermore, cross-reactive antibodies directed against HVR1 peptides of homologous and heterologous strains were generated by these procedures.

Construction and identification of various expression plasmids. pTV2 was constructed from PUC19 as an expression vector for DNA vaccine. This eukaryotic expression vector contains the cytomegalovirus early promoter/enhancer sequence, the simian virus 40 (SV40) replication origin sequence, the adenovirus tripartite leader, and the SV40 polyadenylation sequence. To construct HCV envelope-based DNA vaccine vectors, we replaced the signal sequences of the E1 and the E2 proteins with that of herpes simplex virus type 1 glycoprotein D (gD). This signal sequence has been shown to facilitate the efficient expression and secretion of human immunodeficiency virus type 1 gp160 (1). In addition, C-terminal hydrophobic regions of envelope proteins were truncated to maximize the secretion of these proteins. To construct pSK-s, a PCR fragment that contained a signal sequence of herpes simplex virus type 1 gD (s; amino acid residues 1 to 34) was inserted into pBluescript SK(−) (Stratagene). HCV DNA fragments that encoded amino acid residues 192 to 364 and 384 to 719, which were designated E1t and E2t, respectively, of type 1b (Korean isolate) were amplified by PCR using E1S (5′-CCA GCT TCC AGA TCT GAA GCG CGT AAC-3′), E1AS (5′-GCC GAA TTC TAC ACC ATG GAA TAG TAG-3′), E2S (5′-CCA TAT GCG AGA TCT AGG AGG AAC G-3′), and E2AS

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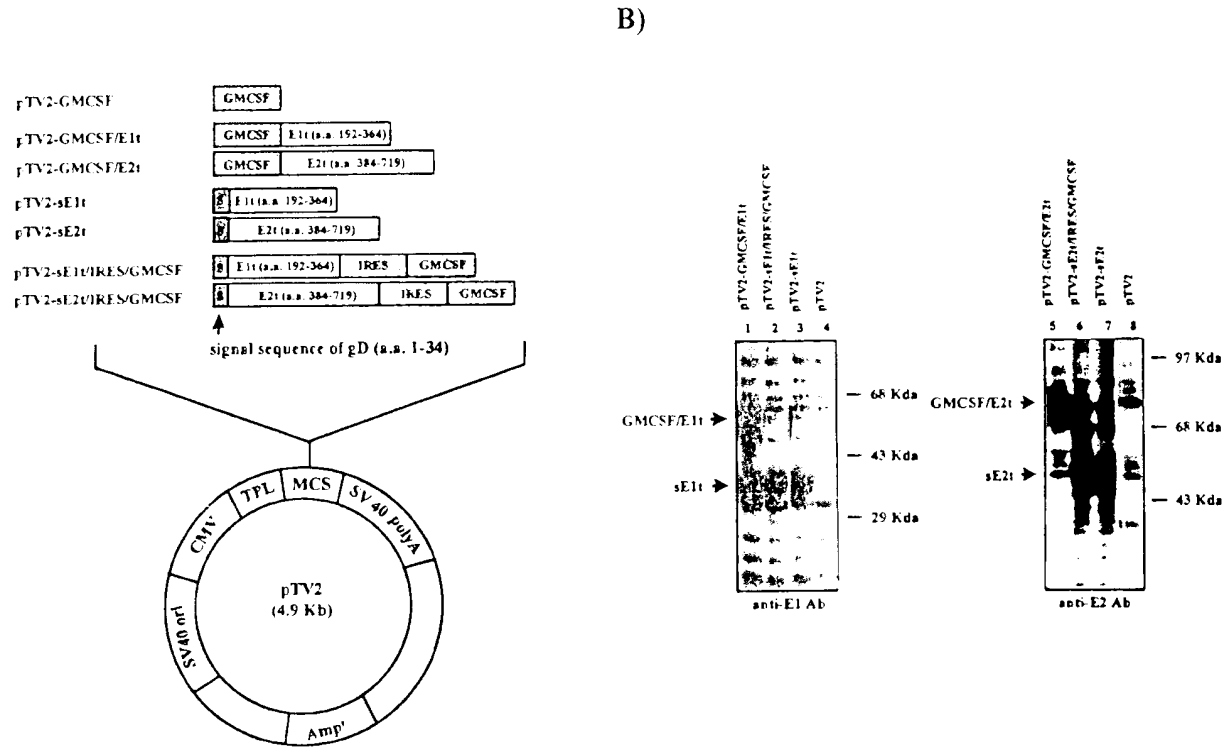

FIG. 1. (NEN Life Science Products) Schematic diagram of expression plasmids used in DNA immunization. All constructs were based on pTV2, represented at the bottom. pTV2-sE1t and pTV2-sE2t were designed to express the C-terminal-truncated E1t and E2t genes, respectively, which encompass the indicated regions, fused with the DNA encoding the signal sequence of gD. pTV2-GMCSF/E1t and pTV2-GMCSF/E2t were also designed to express the E1t and E2t genes fused with that of GM-CSF. pTV2-sE1t/IRES/GMCSF and pTV2-sE2t/IRES/GMCSF were each constructed to express both the HCV envelope and GM-CSF genes under the control of the cytomegalovirus (CMV) promoter and the internal ribosomal entry sequence (IRES), respectively, of encephalomyocarditis virus. pTV2-GMCSF was also made to express the GM-CSF gene alone. Both E1t and E2t genes are shown as open boxes, and the GM-CSF gene and IRES are shown as striped boxes and dotted boxes, respectively. Numbers in parentheses indicate the numbers of the amino acids encoded by the DNA fragment. (B) Identification of HCV envelope proteins. Transfected COS-7 cells were labeled with ^{35}S-Express label (NEN Life Science Products) and immunoprecipitated as previously described. Cell lysates were immunoprecipitated with either anti-E1 (lanes 1 to 4) or anti-E2 (lanes 5 to 8) monoclonal antibodies. Molecular mass markers and corresponding proteins are indicated at the right and left, respectively. Transfected plasmids are indicated at the top of each lane. SV40, simian virus 40; MCS, multiple cloning site; TPL, adenovirus tripartite leader.

(5′-GCG AAT TCT AAT ACT CCC ACC TGA TCG CA-3′) primers. The amplified products were digested with *Bgl*II and *Eco*RI and inserted downstream of pSK-s to produce pSK-sE1t and pSK-sE2t. The resulting plasmids were digested with *Xho*I and *Xba*I and then inserted into these same sites in pTV2 to generate eukaryotic expression vectors pTV2-sE1t and pTV2-sE2t (Fig. 1A). To investigate whether immune responses to HCV envelope proteins are modulated by the codelivery of the GM-CSF gene, we constructed several expression plasmids that carried HCV envelope genes in combination with the GM-CSF gene. A plasmid that expressed GM-CSF but not HCV sequences, pTV2-GMCSF, was constructed by inserting the murine GM-CSF gene for MFG–GM-CSF (6) into pTV2 (Fig. 1A). To construct plasmids that expressed GM-CSF and the HCV envelope proteins as fusion proteins, the GM-CSF gene was amplified by PCR from pTV2-GMCSF with the T7 universal primer and the GC598A primer (5′-CCG CCT CCC ATA TGG CAT TTT TGG ACT GG-3′). These sequences were replaced with hGH of either pSK-hGHE1t or pSK-hGHE2t to produce pSK-GMCSF/E1t and pSK-GMCSF/E2t, respectively. These plasmids were digested with *Eco*RI, and the GM-CSF and HCV sequences were inserted into pTV2 to generate pTV2-GMCSF/E1t and pTV2-GMCSF/E2t, respectively (Fig. 1A). To construct bicistronic plasmids, the GM-CSF gene was amplified by PCR with the GM-CSF N-terminal (GCN) primer (5′-GGA ACC ATG GGG ATG TGG CTG CAG AAT-3′) and the T7 universal primer. The amplified product was digested with *Nco*I and *Bam*HI and inserted downstream of pSK-IRES to generate pSK-IRES/GMCSF. The E1t and the E2t genes were individually inserted into pSK-IRES/GMCSF to produce pSK-sE1t/IRES/GMCSF and pSK-sE2t/IRES/GMCSF, respectively. These plasmids were digested with either *Xho*I and *Xba*I or *Asp*718 and *Xba*I, and the resulting fragments were inserted into pTV2 to generate pTV2-sE1t/IRES/GMCSF and pTV2-sE2t/IRES/GMCSF, respectively (Fig. 1A). These bicistronic plasmids were designed to coexpress each HCV envelope protein and GM-CSF from the same plasmid. To determine if these plasmids expressed immunologically relevant proteins, a transient transfection assay of COS-7 cells was performed as previously described (16, 17). As shown in Fig. 1B, the sE1t and the sE2t proteins were detected at estimated molecular masses of ~34 to 36 and ~49 to 51 kDa, respectively, when cell lysates were precipitated with either anti-E1 or anti-E2 monoclonal antibodies (lanes 2, 3, 6, and 7). It is likely that approximately 5% of the envelope proteins expressed from these constructs were secreted into culture supernatants (data not shown). In addition, the fusion proteins, GMCSF/E1t and GMCSF/E2t, were detected at molecular masses of ~59 to 65 and ~73 to 79 kDa, respectively (lanes 1 and 5). An ~31- to 33-kDa band that corresponded to

TABLE 1. Summary of plasmids injected into rats from different groups,[a] the end point titrations of antibodies in seroconverted rats,[b] and lymphoproliferative responses in immunized rats[c] to HCV E1 and E2 proteins

Plasmids (group)	No. of rats	Titer of[d]		Stimulation index with stimulant[e]:					
				hgh at		hgh-E1t at:		hgh-E2t at:	
		Anti-E1	Anti-E2	1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml
pTV2 (I)	6	NT[f]	NT	0.8 ± 0.06	0.7 ± 0.05	3.1 ± 0.45	3.4 ± 0.49	0.9 ± 0.07	1.2 ± 0.21
pTV2-sE1t + pTV2-sE2t (II)	18	442	5,662	0.8 ± 0.13	0.9 ± 0.07	4.8 ± 1.79	9.8 ± 3.89	1.6 ± 0.84	4.4 ± 1.74
pTV2-sE1t + pTV2-sE2t + pTV2-GMCSF (III)	18	532	8,294	0.9 ± 0.13	0.9 ± 0.10	7.0 ± 3.23	13.0 ± 5.00	2.5 ± 0.57	6.3 ± 2.09
pTV2-GMCSF/E1t + pTV2-GMCSF/E2t (IV)	18	686	16,729	0.8 ± 0.13	0.9 ± 0.11	6.9 ± 1.52	11.9 ± 2.94	2.7 ± 1.39	7.0 ± 3.61
pTV2-sE1t IRES GMCSF + pTV2-sE2t IRES GMCSF (V)	18	1,615	71,647	0.9 ± 0.14	0.9 ± 0.10	7.4 ± 1.46	14.7 ± 6.12	4.6 ± 1.47	11.2 ± 1.81

[a] Female buffalo rats were injected three times with a total of 400 μg of plasmids into the anterior tibialis muscles at 8-week intervals. Two hundred micrograms of pTV2 plasmid was injected into the anterior tibialis muscle of each rat in group I, and 100 μg of each plasmid was injected into each rat in groups II, IV, and V. In group III, 80 μg of each of the pTV2-sE1t and pTV2-sE2t plasmids and 40 μg of the pTV2-GMCSF plasmid were injected.

[b] The anti-E1 and anti-E2 antibody titers were examined with pooled sera of each group of rats at 3 weeks after the last DNA injection (at week 19).

[c] Three weeks after the last DNA injections, each splenocyte obtained from immunized rats (six rats from each group) was used in proliferation assays.

[d] Values are mean stimulation indices for each rat ± standard deviations. Proliferation assays were carried out with triplicate wells.

[e] NT, not tested.

the E1t protein was also observed in pTV2-GMCSF/E1t-transfected cell lysates, presumably due to the cleavage of the junction region between the GM-CSF and the E1t proteins (lane 1). In contrast, a specific protein band was not detected in cell lysates that were transfected with control plasmid pTV2 (lanes 4 and 8). The culture supernatants of transfected COS-7 cells were assayed to determine the expression level of GM-CSF with a commercial GM-CSF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). All plasmids that encoded GM-CSF, including the fusion constructs, produced similar levels of GM-CSF, approximately 5.2 to 6.8 ng/ml (data not shown).

Antibody responses to HCV envelope proteins resulting from DNA immunizations with or without the codelivery of the GM-CSF gene. To elucidate the most effective GM-CSF codelivery method for the induction of immune responses to HCV envelope proteins, the various expression plasmids were injected into the anterior tibialis muscles of female Buffalo rats at 8-week intervals (Table 1). Four- to 6-week-old female Buffalo rats were purchased from Harlan Sprague-Dawley and were housed in the specific pathogen-free facility of the Pohang University of Science and Technology. Briefly, all the rats received intramuscular injections in the anterior tibialis muscle of 200 μg of plasmid DNA dissolved in 150 μl of sterile saline following pretreatment with bupivacaine-HCl (ASTRA) (35). This was followed by two booster immunizations with the same dose at weeks 8 and 16 after the initial DNA injection. Sera were collected by tail bleeding at selected time points and monitored for the presence of antibodies to HCV envelope 1 and 2 (E1 and E2) proteins by ELISA using the hghE1t and the hghE2t proteins that had been purified from recombinant Chinese hamster ovary (CHO) cell lines as specific antigens. Anti-E1 antibodies were initially detected at week 6 postinoculation. The seroconversion rate ranged from 6 to 11% for the different rat groups (Fig. 2A). After a booster immunization, a dramatic increase in the rate of seroconversion was observed for the group V rats (up to 80%). Only slight increases in the seroconversion rate (approximately 21 to 28%) were observed

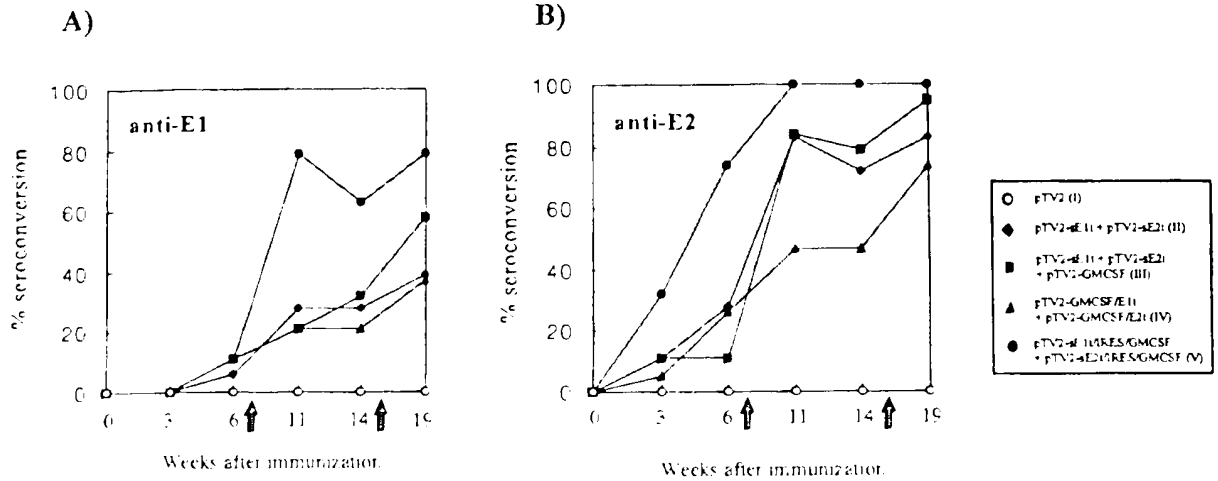

FIG. 2. Detection of seroconversion of rats following DNA immunizations. Female Buffalo rats were injected three times at 8-week intervals and anti-E1 (A) and anti-E2 (B) antibody responses were monitored as described in Materials and Methods. Arrows represent booster DNA injections at weeks 8 and 16. The different plasmids injected are summarized in the text.

for groups II, III, and IV. These rates were further enhanced by a second booster DNA injection at week 16. Final seroconversion rates of 39, 58, 37, and 79% were observed for groups II, III, IV, and V, respectively. In contrast to the responses of the anti-E1 antibody, anti-E2 antibody responses were detectable at week 3, indicating that anti-E2 antibodies were generated earlier than were anti-E1 antibodies in all the envelope DNA-immunized rat groups (Fig. 2B). Among these groups of rats, the group V rats that received bicistronic plasmids showed the highest seroconversion rate: approximately 70% at week 6 and 100% at week 8 when the initial booster DNA was administered. Seroconversion rates of 10 and 30% were observed at week 6 for the group II and the group III rats, respectively, but dramatic increases in seroconversion rates (up to 80%) were observed for both groups soon after the initial booster DNA injection at week 8. A second booster DNA injection at week 16 led to a slight increase in the seroconversion rates for the E2 protein in both groups. In contrast to other groups of rats, the group IV rats that received fusion plasmids had a seroconversion rate of 25% at week 6; this rate gradually increased to approximately 70% upon the administration of two booster DNA injections. These observations that the seroconversion rates were increased by booster DNA injections suggest that primed immune responses appear to be generated and amplified, a process which leads to rapid antibody responses after booster DNA immunizations.

In order to obtain a semiquantitative estimation of anti-E1 and anti-E2 antibody responses, we performed end point titrations by ELISA with serial dilutions of pooled sera obtained from seroconverted rats (18). As shown in Table 1, the antibody titers to HCV E1 and E2 proteins reached 442 and 5,662, respectively, in the group II rats. Slightly higher antibody titers were generated in groups III and IV rats by the codelivery of GM-CSF. As expected, the group V rats had the highest antibody titers to the E1 and the E2 proteins (1,615 and 71,647, respectively). Taken together, our results demonstrate that HCV envelope DNA-based immunization generated antibody responses to E1 and E2 proteins and that the E2t protein was more immunogenic than was the E1t protein. In addition, we found that the codelivery of the GM-CSF gene from plasmids that encoded HCV envelope proteins could modulate antibody responses. The coexpression of GM-CSF and E1t or E2t from a bicistronic plasmid was the most effective delivery method for the enhancement of antibody responses to HCV envelope proteins.

To determine which subclass of anti-E2 immunoglobulin G (IgG) isotypes was induced by HCV E2 DNA-based immunizations with the codelivery of GM-CSF, sera obtained at week 19 were also tested by using horseradish peroxidase-conjugated sheep anti-rat IgG1 (1:500) or IgG2a (1:2,000) secondary antibodies (Serotec). The E2 DNA-based immunizations appeared to predominantly produce IgG2a responses to the E2 protein in all the envelope DNA-immunized rat groups (data not shown). These observations are in agreement with other reports which have shown that intramuscular DNA immunizations preferentially elicited Th-1 type immune responses (9, 23).

Antibody responses to HVR1 peptides. To generate protective immunity against HCV infection, HCV envelope DNA-based immunizations must induce antibodies which are capable of neutralizing viral infection. Due to the lack of an effective in vitro cell culture system for the propagation of HCV, it is difficult to test the neutralizing capability of sera obtained from DNA-immunized rats. It was recently previously reported that antibodies directed against hypervariable region 1 (HVR1) of the E2 protein could have neutralizing capability (38). We tested the ability of sera obtained from rats that were injected with GM-CSF and HCV envelope DNA to bind to HVR1 peptides. Various HVR1 peptides (Fig. 3A), including type 1b (HCV-K and HCV-J4; Korean and Japanese isolates, respectively) and type 1a (HCV-H), were purchased from the PeptidoGenic Research Co. (Livermore, Calif.). HVR1-specific antibodies were analyzed with serum samples from DNA-immunized rats by ELISA. Briefly, different HVR1 peptides (2 μg/ml) which were biotinylated at their N termini were coated overnight at 4°C following precoating treatment with streptavidin (2 μg/ml) for 2 h at room temperature. After a blocking with bovine serum albumin for 1 h, 100 μl of test sera (1:100 dilution) was added to each well and incubated for 1 h at room temperature. Bound antibodies directed at the HVR1 peptide were also detected with horseradish peroxidase-conjugated sheep anti-rat IgG (1:3,000 dilution) antibodies. Compared with that from the pTV2-immunized control group, sera from groups II, III, and V rats showed the ability to bind strongly to a homologous (HCV-K; type 1b) HVR1 peptide (amino acids [aa] 384 to 403) (Fig. 3B). In contrast to what was found for the total anti-E2 antibody titers, the levels of antibody responses to HVR1 were similar for groups II, III, and V, which indicated that GM-CSF codelivery had little effect on the generation of antibody responses to HVR1. The lower level of binding affinity of sera from GM-CSF/E2t DNA-immunized rats (group IV) may be due to a block of the HVR1 epitope by its N-terminal fusion with the GM-CSF protein. We also examined whether anti-HVR1 antibodies obtained from DNA-immunized rats had cross-reactivity to HVR1 peptides of heterologous strains. It was previously reported that isolate-independent anti-HVR1 antibodies seemed to map to the C terminus of HVR1 (39). Therefore, we tested the abilities of sera obtained from group I and group V rats to bind to various HVR1 peptides which did (aa 384 to 410) or did not (aa 384 to 403) express the C-terminal end of HVR1. Cross-reactive antibodies which were capable of binding to heterologous HVR1 peptides were generated by the HCV envelope DNA immunizations. Interestingly, heterologous HVR1 peptides which expressed the C-terminal end of HVR1 (aa 384 to 410) cross-reacted with the rat sera (Fig. 3C). However, heterologous HVR1 peptides that lacked this region (aa 384 to 403) did not cross-react or cross-reacted only slightly (data not shown). In addition, HVR1 expressed by the same genotype (HCV-J4; type 1b) reacted more strongly than did HVR1 expressed by a different genotype (HCV-H; type 1a). This is likely due to the higher degree of conservation of the C terminus of HVR1 between HCV-K and HCV-J4 (Fig. 3A). Sera from other groups of rats showed similar abilities to bind to heterologous HVR1 peptides, although the relative values of optical density at 405 nm for each group of rats varied. These results strongly suggest that HCV envelope DNA-based immunizations can generate cross-reactive antibodies to the HVR1s of various HCV strains.

Enhancement of lymphoproliferative responses by the codelivery of the GM-CSF gene. To investigate the effect of GM-CSF codelivery on the induction of the cellular immune response, the lymphocyte proliferative assay was performed as described previously (17). At 2 or 3 weeks after the final DNA inoculation, splenocytes were tested for their proliferation in response to stimulation with specific antigens. The isolated spleen cells were resuspended to a concentration of 3×10^6 cells/ml. A 100-μl aliquot was added to each well of a 96-well microtiter round-bottomed plate. Recombinant proteins were added to the wells in triplicate at a final concentration of 1 or 10 μg/ml. To assure that the spleen cells were healthy, concanavalin A (5 μg/ml) was used as a positive mitogenic control.

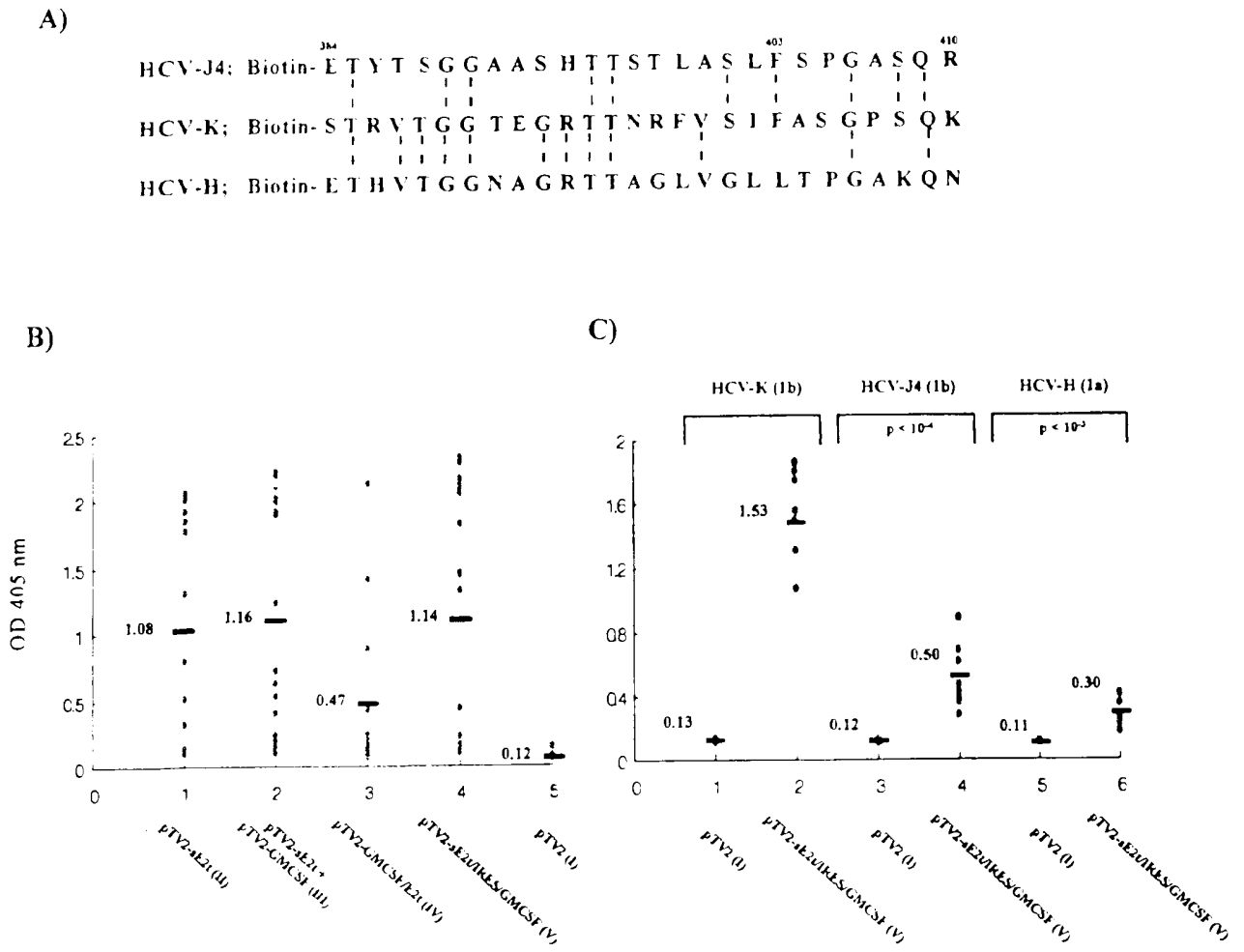

FIG. 3. Antibody responses directed to homologous and heterologous HVR1 peptides. Various HVR1 peptides which were biotinylated at their N termini were used in an ELISA. Sera bled at week 19 (1:100 dilution) were examined. In panels B and C, the optical density (OD) value at 405 nm for each seroconverted rat in the group is shown; average values are indicated by horizontal lines. The different plasmids injected are indicated at the bottom. (A) Comparison of various HVR1 sequences of different HCV strains. Amino acids that are the same for different HVR1 peptides are indicated by dashed lines, and the numbers of amino acids in the HCV polyprotein are indicated. (B) Abilities of sera obtained from DNA-immunized rats to bind to homologous HVR1 (HCV-K) peptide (aa 384 to 403). (C) Abilities of sera to bind to homologous and heterologous HVR1 peptides (aa 384 to 410). Statistical analysis was performed by the Student *t* test.

Lymphocytes from all rat groups did not have specific proliferative responses when a human growth hormone (hgh) protein, a negative control antigen, was used (Table 1). Spleen cells obtained from the group II rats had stimulation indices of approximately 4.4 and 9.8 with the addition of hghE2t and hghE1t proteins, respectively. When codelivered with the GM-CSF gene, HCV envelope DNA elicited higher lymphoproliferative responses to HCV envelope proteins than it did when administered without the GM-CSF gene to group II rats. These responses increased in a dose-dependent manner, and the peak stimulation index was approximately 6.3 to 7.0 for hghE2t and 13.0 to 11.9 for hghE1t for both group III and IV rats. However, a lymphoproliferative response was not detected by the addition of the hghE2t protein in the group I rats that received vector DNA alone. Although there were measurable lymphoproliferative responses to hghE1t in the group I rats, the responses were not dose dependent, which indicated that they were not antigen specific. Interestingly, the bicistronic coexpression of GM-CSF and HCV envelope proteins significantly enhanced lymphoproliferative responses to both hghE1t and hghE2t proteins, which reached stimulation indices of 14.7 and 11.2, respectively. These results indicate that the codelivery of the GM-CSF gene enhances T-helper cell responses to HCV envelope proteins in DNA-based immunization.

Conclusions. In this study, we described several observations concerning DNA immunizations using HCV envelope expression plasmids with or without the use of GM-CSF as a molecular adjuvant. For example, the immunogenic potential of the HCV E2t protein is likely to be higher than that of the HCV E1t protein, at least in our experimental conditions. This observation is compatible with the results of Lanford et al. (15), who observed a lower level of reactivity of anti-HCV-positive human sera to insect cell-expressed E1 protein (4 of 18 sera positive) than to analogously expressed E2 protein (15 of 18 sera positive). In addition, we have observed that both the humoral and the cellular immune responses to HCV envelope proteins were augmented by the codelivery of the GM-CSF

gene. The antibody and lymphoproliferative responses to the E2 protein were increased approximately 3.5- to 12.7-fold and 1.4- to 2.5-fold, respectively, by the codelivery of the GM-CSF gene. Inoculations of bicistronic plasmids elicited higher levels of antibody and lymphoproliferative responses than did the coinoculation of two independent expression plasmids that encoded the GM-CSF gene and each HCV envelope gene. These data suggest that the coexpression of GM-CSF and the envelope proteins from the same plasmid may optimize immune responses to the HCV envelope proteins. We suggest that the local concentration of GM-CSF may be one of the critical factors that contribute to the augmentation of immune responses to the coexpressed antigens. This model is partially compatible with the findings of Xiang et al. (36), who observed that the separate inoculation of the GM-CSF gene and antigen-encoding plasmids several hours apart had no effect on the magnitude of antigen-specific antibody responses. In addition, immunization with GM-CSF–envelope fusion constructs appeared to induce smaller immune responses than did those with bicistronic constructs. It is possible that the biological activity of GM-CSF may be altered when it is fused to the HCV envelope proteins. We conclude, therefore, that the bicistronic coexpression of GM-CSF and antigens is the most effective way to induce immune responses for the HCV envelope DNA-based immunizations.

It has been suggested that the HVR1 of the HCV E2 protein is comparable to the V3 loop of human immunodeficiency virus type 1 which contains a neutralizing determinant (22, 27, 34). Recently, it was shown that hyperimmune serum raised against a synthetic HVR1 peptide induced protection against homologous HCV infections in chimpanzees (7). In addition, the early appearance of antibodies that were directed against the N terminus of HVR1 is associated with acute self-limiting infections of HCV (39). Our experiments demonstrate that strong antibody responses to a homologous HVR1 peptide were induced in DNA-immunized rats. The levels of anti-HVR1 antibodies that were generated from groups II, III, and V rats were similar (Fig. 3B), which is likely due to the highly immunogenic potential of HVR1. In addition, we demonstrated that cross-reactive anti-HVR1 antibodies which mainly recognized the C-terminal end of the HVR1 were generated in DNA-immunized rats. These observations are consistent with clinical results that were obtained from acute and chronic HCV-infected patients. Cross-reactive antibodies directed to HVR1 have been observed during chronic HCV infection (29). These isolate-independent antibodies were shown to react to the C terminus of HVR1 (39). It is notable that HCV E2 DNA-based immunization can generate cross-reactive antibodies to heterologous HVR1, as is seen in natural infections of HCV. Although further studies concerning the neutralizing capability of these antibodies raised by DNA immunization are necessary, our studies suggest that immunization with plasmid DNAs that express HCV envelope proteins could result in the generation of protective immunity against heterologous HCV challenge.

S.W.L. and J.H.C. contributed equally to this work.

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